

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
9 October 2003 (09.10.2003)

PCT

(10) International Publication Number
WO 03/083477 A2(51) International Patent Classification⁷: G01N 33/543

(21) International Application Number: PCT/HU03/00025

(22) International Filing Date: 28 March 2003 (28.03.2003)

(25) Filing Language: Hungarian

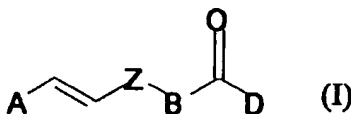
(26) Publication Language: English

(30) Priority Data:
P 0201091 29 March 2002 (29.03.2002) HU(71) Applicants (for all designated States except US): COM-
GENEX RT. [HU/HU]; Bem Rpt. 33-34., H-1027
Budapest (HU). MTA SZEGEDI BIOLÓGIAI KU-
TATÓKÖZPONT [HU/HU]; Temesvári Krt. 62., H-6726
Szeged (HU).

(72) Inventors; and

(75) Inventors/Applicants (for US only): DARVAS, Fer-
enc [HU/HU]; Zápor U. 11/B., H-1034 Budapest (HU).
ŰRGE, László [HU/HU]; Kerékgyártó U. 3., H-1147
Budapest (HU). BUCSAI, Ágota [HU/HU]; Kerék U. 26.,
H-1035 Budapest (HU). BÉRES, Mariann [HU/HU];
Szépvölgyi U. 212., H-1025 Budapest (HU). DORMÁN,
György [HU/HU]; Kondorosi Út 80., H-1119 Budapest
(HU). KRAJCSI, Péter [HU/HU]; Batty u. 7., H-1025
Budapest (HU). GÖDÖRHÁZY, Lajos [HU/HU]; Mec-
seki U. 49., H-2030 Érd (HU). BÁGYI, István [HU/HU];
Rákóczi Út 16., H-1161 Budapest (HU). PUSKÁS, László[HU/HU]; Fiume U. 13., H-6753 Szeged (HU). HACK-
LER, László [HU/HU]; Molnár U. 6., H-6723 Szeged
(HU). ZVARA, Ágnes [HU/HU]; Remény U. 38., H-6725
Szeged (HU).(74) Agent: KORMOS, Ágnes; Váci Út 66., H-1132 Budapest
(HU).(81) Designated States (national): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE,
SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ,
VC, VN, YU, ZA, ZM, ZW.(84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,
ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO,
SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM,
GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished
upon receipt of that reportFor two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.(54) Title: NEW ACTIVE SOLID SUPPORT AND METHOD FOR SURFACE IMMOBILIZATION OF COMBINATORIAL
COMPOUNDS OR LIBRARIES(57) Abstract: The subject of the invention is a method for production of an ac-
tive solid support suitable for anchoring drug molecules through covalent bonds,
using the following methods a.) a solid support prepared in advance, preferably by
pre-treatment in the presence of strong bases or acids, then reacted with a polyamine-
functionalized alkoxy-silane derivative, preferably 3-[2-(2-aminoethylamino)ethy-
lamino]propyl trimethoxy-silane, followed by washing and drying, b1) the solid sup-

port obtained in the procedure described above in Section a.) will be reacted with (I) where Z: = bond or a -CO or -CNR 1 group and group 121 is an alkyl, aralkyl or aryl group; B: = bond or a $-(CH_2)_n$ or $-(CH_2)_n$ O- $(CH_2)_n$ or O- $(CH_2)_n$ - group; A: = a branched or unbranched alkyl or substituted aryl or aralkyl group containing 1-5 carbon atoms; D: = a halogen or -O-A group and n = 0, 1, 2, ... integer - with a bi-functional reagent, preferably reacted with acryloyl/chloride or 1,4-butanediol diacrylate in the presence of an organic or inorganic scavenger or b2) the support prepared using the method described in part (a) is reacted with a bi-functional reagent, preferably 1,4-butanediol diglycidyl-ether or epichlorohydrin, in the presence of an organic or inorganic scavenger contain-
ing terminal epoxy groups and an amino-group reactive functional subgroup. The invention also includes the active solid support produced using the several methods described above, as well as application of the active solid support described above for production of chemical microarrays and combinatorial chemical microarrays. The invention further comprises a method for immobilization of what have been termed organic molecules of low molecular weight, having a size less than 1500 Da, preferably between 250 and 750 Da. This is achieved by anchoring an unmodified drug or drug candidate molecule to the active solid support described above by adding the appropriate functional groups of the drug or drug candidate molecule, preferably executed using a micron-sized robotized binding technique.

New active solid support and method for surface immobilization of combinatorial compounds or libraries

This invention is a new synthetic method of immobilizing drugs and drug candidates, as well as generally biologically active molecules, so that their state of biological activation and binding site selectivity remains unaltered.

To achieve these goals, this invention provides a method to synthesize a new solid support or support group, which can bind drugs and drug candidate molecules – in scientific terms immobilization or anchoring – namely, through the use of reactive groups placed on linkers with branched structures.

Our newly invented supports are capable of covalently anchoring small molecules having no linkers and/or molecules that contain linkers of various lengths and reactive groups. Such solid supports can be used to create various small molecule arrays, as well as their applications in molecular fields of agrochemistry, biology, biotechnology and pharmacology.

Scientific Background

It is a well-known fact that as a result of the completion of the sequencing stage of the human genome project, several thousands of new proteins are yet to be classified. Identifying which of these proteins constitute potential drug targets shall be an important challenge for drug researchers in the years to come. Numerous methods of relating these identified proteins to specific diseases have been developed, such as the 2D electrophoresis comparison method (A. Görg, *Proteomics*, 2000, July, 3) and isotope labeling, using methods combined with mass spectrometry (S.P. Gygi et al, *Proteomics*, 2000, July, 31).

In many instances, these methods do not produce the desired results: in cases when proteins are present only in low concentrations, for example, or when a vast number of proteins must be separated, detected and resolved. Furthermore, identifying proteins associated with diseases is not enough, since the true goal of drug research is to discover small molecules which influence the functions of these proteins.

The binding of small molecules to a specific protein validates its druggability, by demonstrating whether the small molecule is capable of binding to it at all. Furthermore, subsequent protein expression studies can be used to determine whether the disease state can be influenced by this binding, which also verifies the function of the protein (G. Dormán, et al. Current Drug Discovery, 2001, 1, 21-24).

With the emergence of new developments in combinatorial chemistry, where every combination of reagents are reacted with and bound to a central structural element, large numbers of small molecules, as well as large numbers of analogues of previously known effective drugs, can be manufactured.

As a result of the above findings, the effective identification and classification of new and known proteins, through affinity-based interactions with large numbers of small molecules, has come to the forefront of scientific investigations. Since the method mainly allows the identification of proteins coded by newly discovered genes, and since their functional validation is performed mainly by using synthetically prepared small organic molecules, this new approach is called chemical genomics and proteomics.

Affinity-based methods for identifying and isolating proteins were previously used for immobilizing small molecules on cellulose and various polymers. The protein mixture is streamed through the resulting affinity columns. The proteins then leave the columns at various speeds, according to their binding affinity toward the immobilized small molecules, and are thus separated from the non-binding or hardly-bound proteins.

A number of attempts have been made to increase the throughput of this method but these attempts have brought little progress. Collectively known as affinity chromatography, these methods involve time-consuming procedures, require large samples of the small molecules, and do not allow for easy parallel application.

The high density anchoring of small molecules to miniaturized chips has brought revolutionary change to the field. On such a chip, the molecules are placed in a two-dimensional array formation. The term "microarray" is used in the following description of our invention to denote a miniaturized planar array of small, immobilized molecules. The advantage of microarrays over classical high-throughput biological screening is that the anchored compounds are arranged adjacent to each other, so that a large number of measurements under identical conditions can be carried out, allowing for more accurate comparison. A further advantage is that the microarray can be combined with high-capacity robotized technologies.

The technology of immobilization on cellulose or glass surfaces was first developed for production of DNA microarrays, and was applied in a wide range of hybridization experiments. Several methods were developed to immobilize DNA molecules and oligonucleotides (S.L. Beaucage, *Current Med. Chem.*, 2001, 8, 10, 1210). The above technology had a great impact on the completion of the Human Genome Project. Later, the development of protein microarrays was begun in order to determine protein-protein interactions and protein expression levels (B.B. Haab, *Curr. Op. Drug Disc. Dev.*, 2001, 116-123).

M. Beier and J.D. Hoheisel (*Nucleic Acids Res.* 27(9) 1970-1977 (1999)) describe a solid support having a branching structured linker system for covalent attachment of DNA. The derivatization of the surface and the build-up of the linker system consist of four reaction steps. The activity of the surface is enhanced prior to the immobilization with heterobifunctional linker molecules such as PDITC (phenylendiisothiocyanate), DSC (disuccinimidylcarbonate), or DMS

(dimethylsuberimidate). Different types of DNA molecules may thus be effectively anchored to the surface but the stability of the immobilization of each is different. Moreover, these differences allow us to draw the conclusion that the covalent bond formed between the surface and amino groups of the DNA molecule is weak. Consequently, this particular surface may be used to anchor small molecules only with low efficiency.

Attempts to immobilize small molecules have been made only recently. These immobilization techniques use different anchoring strategies and various chemical mechanisms. The anchoring of sample molecules may be different in terms of both efficiency and stability. As an analogue of DNA microarrays, the most preferred support for small molecule immobilization is a glass microscope slide. One anchoring technique was described by Graffinity (Germany), in which organic molecules are immobilized on a gold surface via thiol functional groups (DE 100 27 397).

Researchers have developed several chemical reagents for immobilizing small molecules (JP 3 032 740 or WO-01/01143 USA). At present, chemically derivatized glass microscope slides are used for hybridizing chemical arrays. Small molecules are often equipped with a functional group so that they are able to anchor to the surface of the support. A functional group may be a terminal thiol, amino or carboxyl group introduced through a linker arm. The modified small molecule may anchor to a surface bearing a disulphide, maleimide, amino, carboxy, ester, epoxy, bromo-cyanide or aldehyde functional group. Binding occurs via the formation of thioether, ether, amide or amine bonds between the small molecule and the surface. In one method (USA 5 919 523), arrays of peptides, oligonucleotides, small organic molecules, and ligands are produced by immobilizing them on solid supports covered by either glycane or polymers used in solid phase synthesis. The bond is via amino, carboxy and/or hydroxy groups of the polymer.

The drawback of methods using mercapto or epoxy silanized surfaces (USA 5 919 626) is that immobilized molecules lie too close to the surface, which causes poor accessibility for the probe molecules and, consequently, a decrease in number the number of specific bonds formed.

In another technique, combinatorial synthesis of molecules *in situ* on a polypropylene surface was carried out, such that the position of the synthesized molecules was known. Later, this array was used in biological screening assays (D. Scharn et al., J. Comb. Chem., 2000, 2, 361).

With the split-mix method of combinatorial chemistry (A. Furka et al., Int. J. Pept. Protein Res, 1991, 37, 478), quite a large number of compounds can be produced in mixture. These compounds can then be anchored effectively onto a glass surface using a thiolreactive anchoring group (maleimid) using a technique called "microprinting" (G. MacBeath, et. al., J. Am. Chem. Soc., 1999, 121, 7967). Although this method can be applied to determine the interaction of a vast number of compounds with proteins, the anchoring of the compounds happens randomly, thus determining the structure of a compound in a given position of the array is complicated.

Developing microarrays for combinatorial chemistry is based on applying solid supports, which can anchor various small molecules. These anchored synthetic molecules have numerous advantageous applications. The combinatorial chemical microarrays may potentially become important tools in molecular biology and drug development research.

These new tools and methods can be applied to determining the interaction of known drugs with newly discovered proteins, and therefore to classification of these proteins. In this case, the specifically binding proteins can be separated from the non-binding ones, removed from the chip, and then identified using an appropriate method (MS-MS; M.J. Dutt and K.H. Lee. Proteomic analysis, Current Opinion in Biotechnology, 2000, 11, 176-179).

This method can also be applied to high throughput biological screening, when the effective compounds emit fluorescent signal in a given position of the array. Since positions within the microarray have been mapped, the structure of the active compounds can be easily identified.

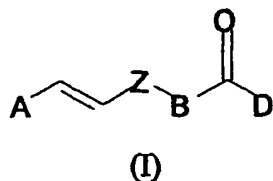
Description of the invention

As opposed to the limiting factors and specialized methods involved in all the currently known methods, there is a need for a new technologically simple approach, whose process of binding small molecules to a given surface is chemically compatible with the characteristics of the widest range of biologically active organic compounds (for example drug and drug-like molecules). This way, such an approach can be used in techniques based on various biological interactions, such as in detecting proteins, allowing for the production of a microarray capable to identify molecules showing potential biological activity. For application purposes, it is desirable that the chemical anchoring group also be able to bind organic molecules directly, and not only via terminal functional groups of specific side chains.

Based on the above observations, this invention consists of a method for producing an active solid support that is suitable for anchoring drug molecules by covalent bonds, by the following methods:

a.) a solid support, prepared ahead of time, preferably by pre-treatment executed in the presence of strong bases or acids, is reacted with a polyamine-functionalized alkoxy-silane derivative, preferably 3-[2-(2-aminoethylamino)ethylamino]propyl-trimethoxy-silane, and then washed and dried,

b1) the solid support obtained in the procedure described in Section a.) is reacted with (I)



— where

Z: = bond or a -CO or -CNR₁ group, where R₁ is an alkyl, aralkyl, or aryl group;

B: = bond or a -(CH₂)_n- or -(CH₂)_n-O-(CH₂)_n- or O-(CH₂)_n- or -NH-(CH₂)_n- group;

A: = branched or unbranched alkyl or substituted aryl or aralkyl group containing 1-5 carbon atoms;

D: = halogen or -O-A group and

n = 0, 1, 2, ... integer — with a bi-functional reagent, preferably reacted with acryloyl/chloride or 1,4-butanediol diacrylate in the presence of an organic or inorganic scavenger

or

b2) the support prepared using the method described in part (a) is reacted with a bi-functional reagent, preferably with 1,4-butanediol diglycidyl-ether or epichlorohydrin in the presence of an organic or inorganic scavenger, which contains terminal epoxy groups and an amino-group reactive functional subgroup.

In a useful variation of the invention, glass, polystyrol or polypropylene chips, preferably glass microscope slides, are used as the solid support.

The invention furthermore consists of the active solid support produced using the various methods described above.

A further objective of the invention is to apply the active solid support described above for the production of both chemical and combinatorial chemical microarrays.

An additional objective of the invention is to provide a method for immobilizing organic molecules of what is termed "low molecular weight," having a size of 1500 Da or lower, preferably between 150 and 750 Da. This is achieved by anchoring an unmodified or tethered drug or drug candidate molecule to the active solid support described above by adding the appropriate functional groups of the drug or drug candidate molecule, preferably executed by a miniaturized robotized binding technique.

In another useful variation of the invention, the microarray, produced using the active solid support and the procedure described above, is used for quick execution of agrochemical, biotechnological, biological, or medicinal chemical tests, and especially for studying the interactions between small molecules and proteins.

Another objective of this invention is to use the microarray, produced using the active solid support and the procedure described above, for identifying new proteins, for rapid analysis of the druggability of proteins, for determining secondary binding sites, for identifying nucleic acids (RNA, DNA) that bind small molecule ligands, for identifying protein-protein, protein-DNA, protein-lipid, protein-nucleotide, protein-carbohydrate, protein-vitamine, and protein-metal ion complexes, or for identifying ligands and/or metal ions.

A further objective consists in the use of the microarray, produced using the active solid support and the procedure described above, for biological screening assays, in which the microarrays, containing immobilized ligands, and known or unknown proteins or complexes of these proteins formed using biomolecules, are used in either labeled (for example with fluorescent tag) or unlabeled form, or labeled or unlabeled complexes of known or unknown nucleic acids are used, or ions or their

complexes are used, or eukaryotic or prokaryotic cells are used, after which the quantity and quality of the bound proteins, nucleic acids, ions, or cells is determined using suitable analytical technologies.

Another objective of the invention is to use the microarray, produced using the active solid support and the procedure described above, for determination of anchoring kinetics, balances and kinetic constants, and balance constants.

Furthermore, the procedure we have invented may be used to achieve quick and inexpensive immobilization and anchoring of small molecules used as drugs or considered as drug candidates that do or do not contain branched linkers.

A covalent bond forms between the new solid support we have invented and the molecules to be anchored. The covalent bond results in high stability and constant surface concentration. As to terminology, the molecules anchored to the surface are called sample molecules, while the compounds that react with those molecules are called probes.

Classic methods, which anchor the probes to the surface of the solid support through covalent bonds, use molecules which contain various chemical modifications (in the case of nucleic acids this refers to amino carboxyl, or thiol derivatives, while in the case of small molecules this refers to linkers containing carboxyl-, amino-, or thiol-terminal groups), while active aldehydes or such other groups, which are able to react with the above terminal groups are located on the surface.

The method described as part of this invention, on the other hand, does not require chemical modifications of the probes to be anchored. This approach significantly reduces the costs involved in high throughput production. The anchoring does not require further chemical reactions, as opposed to the methods mentioned above such as reduction of the newly formed bonds. Therefore, it is an advantage that the samples containing chemical groups, sensitive to reduction, can be anchored to the solid structure without damage or modifications to the structure.

In another useful variation of our invention, synthetic molecules containing amino, imino, or thiol functional groups or heterocyclic nitrogen are anchored to a solid support that has reactive acrylic functional groups on its surface.

Another variation of our invention involves the anchoring of synthetic molecules containing amino or thiol functional groups to a solid support that has reactive epoxy functional groups on its surface. The functional groups are anchored in high density to the solid support via branching, non-flexible or conformationally flexible linker arms enabling high-immobilized molecule concentration on the surface. Another advantage of the system is that the linker arms give small molecules better access to the sample and allow for the creation of stable covalent bonds between the active surfaces and the drug or drug candidate molecules.

A further advantage of our invention is that the hydrophobic properties of the surface can be modulated, which in turn directly affects the density of the microarray produced. When a hydrophilic surface is used to produce microarrays, larger spots are formed, resulting in a microarray of lower density (100-2000 spots/microarray). The hydrophilic property of the surface can, however, be helpful in affinity experiments because the probe molecule does not bind to the surface aspecifically. The spots generated using a hydrophobic surface are smaller, hence the density of the microarray is higher (2000-6000 spot/microarray), though in certain affinity experiments this may be less desirable.

The method described herein specifies the use of properly pretreated solid supports (described below), such as glass, polypropylene or polystyrene slides, preferably glass microscope slides. In our own experiments testing solid supports, commercially available aldehyde-coated slides were used (SuperAldehyde Cat. No. SMA-25, Arrayit, USA). The procedure for pretreating the slides includes two steps: (i) etching in NaOH and (ii) washing in distilled water.

Another useful variation of our invention includes derivatization of the pretreated solid supports in two reaction steps. In the first step, the supports are reacted, preferably with 3-[2-(2-aminoethylamino)ethylamino]propyl-trimethoxysilane. Free amino functional groups are introduced to the surface during this step. In the second reaction step, the triamino-silanized surface is treated with acryloyl-chloride. The result is a molecule having branching carbon chain and amide-bonds, and a structure that includes reactive acryl groups. Alternatively, in the second step, the surface can be modulated to hold active epoxy groups and have either hydrophobic or hydrophilic properties. To produce a hydrophilic or hydrophobic epoxy surface, supports are treated with 1,4-butanediol diglycidyl ether or epichlorohydrin, respectively, in the second reaction step. In these cases, epoxy groups are introduced into the positions of the amino groups in the branching structure.

In one other useful variation of our invention, glass slides pretreated with NaOH and water (see above) are reacted with 3-[2-(2-aminoethylamino)ethylamino]propyl-trimethoxysilane in an alcohol solution, preferably a 95% aqueous solution of ethanol or methanol, in the first step of derivatization. Later, the slides are washed using the same alcohol and water and dried at 100-110°C.

In the second step, in order to introduce amide-bonds, the slides are treated with acryloyl-chloride in a non-polar solvent, preferably dichloroethane, dichloromethane or something similar, in the presence of an equivalent amount of a base such as pyridine, diisopropylethyl-amine, or another similar base. Finally, the slides are washed using the applied solvent and dried.

In the second step, where an epoxy-activated surface is prepared, the slides are treated with 1,4-butanediol-diglycidyl-ether in short carbon chain alcohol containing a dissolved base, preferably in ethanol containing NaOH. Alternately, the slides may be treated with epichlorohydrin in a non-polar solvent containing a dissolved base,

preferably chloroform containing pyridine. Later, the resulting hydrophobic slides are washed with the particular solvent used and dried.

All reagents used in our invention are commercially available.

The properties of solid supports derivatized using the method described herein allows for immobilization of synthetic molecules at a high density and in an ordered pattern with high stability for use in chemical microarray technology.

The solid supports produced using the method we have developed are especially suitable for the preparation of chemical microarrays by means of immobilization of small organic compounds. The produced microarrays can be used for investigating interactions between (i) small molecules and proteins, (ii) small molecules and nucleic acids, (iii) small molecules and biomolecules of low molecular weight, or for protein detection based on biochemical interactions, or for high throughput screening of biologically active drug-like molecules. The invention is also appropriate for proteomic studies, combinatorial analyses of different nucleic acids, proteins, and molecules of low molecular weight. The invention may be used in chemistry, molecular biology, biotechnology, agrochemistry, proteomic studies, and drug development.

The most generally used method for protein detection involves labeling with fluorescent dyes or the use of fluorescent proteins in high-throughput biological screening. The method of immobilization we have developed may be applied to fluorescent detection and to other new detection techniques, such as surface plasmon resonance (DE 100 27 397) and planar waveguide (WO-0155691) combined with fluorescent labeling. These techniques detect small changes in surface properties corresponding to biological interactions.

To produce chemical microarrays using the method we have developed, unmodified, biologically active molecules, potentially biologically active molecules, or small molecules equipped with a linker arm are anchored to the solid supports activated as described above. Molecules are dissolved in a highly polar solvent

(preferably dimethylformamide, dimethylsulphoxide or water), printed on the surface, incubated for 1-3 hours, then dried.

Preparations for and application of the invention are demonstrated in the following figures and examples.

I. Figures

Figure 1.: Chemical arrays prepared by manual pipetting using three developed support surfaces (a: an acrylic surface, b: a hydrophilic epoxy surface, c: a hydrophobic epoxy surface). In the figure, the two lower spots represent samples containing a pH buffer, while the two upper spots represent samples without a pH buffer.

Figure 2.: Arrays prepared by robotic printing using three developed support surfaces (a: an acrylic surface, b: a hydrophilic epoxy surface, c: a hydrophobic epoxy surface). 1-6: concentrations of the applied biotin solutions.

Figure 3.: Arrays prepared by robotic printing using three developed support surfaces (a: an acrylic surface, b: a hydrophilic epoxy surface, c: a hydrophobic epoxy surface). 1-6: concentrations of the 4-amino-benzamidine solutions used.

II. Examples

Example 1.

Preparation of an acryl surface

Pretreatment of the glass microscope slides

Commercially available, non-derivatized glass microscope slides were etched in a 10% NaOH-solution (Source of NaOH: Molar Chemicals Ltd., Hungary, purity >98.5%) for 24 hours, then washed with water, neutralized with a 1% solution of HCl, washed again with water until pH-neutral, then dried.

step a.) Preparation of a triamino silanized surface

The previously activated glass slides are treated with a 3% solution of 3-[2-(2-aminoethylamino)ethylamino]propyl-trimethoxysilane (ICN Biomedicals Inc., Aurora, Ohio) in 95% aqueous methanol for 2 hours. Later, the slides were washed using methanol, then water, and were finally dried and incubated at 105°C for 15 minutes.

step b.) Preparation of a reactive acrylic surface

The silanized slides obtained in step a.) were incubated for 2 hours with 30 mmol acryloyl-chloride (ICN Biomedicals Inc., Aurora, Ohio) and 30 mmol diisopropylethyl-amine (ICN. Biomedicals Inc., Aurora, Ohio) dissolved in 62.5 mL dichloroethane (DCE) (Merck, Germany). Later, the slides were washed with DCE and dried at room temperature.

Example 2.

Preparation of a hydrophobic epoxy surface

Slides obtained from Example 1, step a.) were incubated for 2 hours with 30 mmol epichlorohydrin (Fluka, Germany) and 12 mmol (1 mL) pyridine (Fluka, Germany) dissolved in 66.7 mL chloroform (Molar Chemicals Ltd., Hungary, purity >99%). Later, the slides were washed with chloroform and dried at room temperature.

Example 3.

Preparation of a hydrophilic epoxy surface

Slides obtained from Example 1, step a.) were incubated for 2 hours with 30 mmol 1,4-butanediol diglycidyl ether (Fluka, Germany) and 5 mmol NaOH dissolved in 63 mL ethanol (Molar Chemicals Ltd., Hungary, purity >99.7%). Afterwards the slides were washed with ethanol and dried at room temperature.

Example 4.

Preparation of high density chemical microarrays

In our experiments, a solution of biotin having an amino linker arm was introduced to the developed surfaces, either manually or using a MicroGrid Total Array System spotting robot (BioRobotics, England). The solvents used in both experiments were: dimethyl formamide, dimethyl sulfoxide, and water. Biotin solutions were introduced in several concentrations in a pH 10 phosphate buffer or without any pH buffer. The slides were incubated for 2 hours at room temperature in a humidity-controlled chamber in order to prevent evaporation of sample droplets. Using manual pipetting or robotic printing, the introduced sample volume is 1-0.5 μ L or 1 nL, respectively. After sample immobilization the slides are washed using an aqueous solution of 1 x SSC (0.1 M NaCl, 15 mM tri-sodium-citrate), 1% BSA (bovine serum albumin), and 0.2 w/w% SDS (sodium-dodecil-sulfate) and then water. Later, the slides were dried at room temperature and stored at ambient temperature in the dark.

Example 5.

Protein affinity experiments

Arrays prepared in Example 4 were reacted with 10 μ L of Cy3 streptavidine (10 mg/L) under a glass coverslip for 1 hour at room temperature. Then the slides were washed in three steps as follows: Step 1. washing using PBS (phosphate buffer, pH 7.0), Step 2. washing using 0.2 x SSC, Step 3. washing using 2 x SSC. After the slides

had dried, they were scanned using a GSI Lumonics, Scannaray Lite confocal laser scanner. Results are shown in Figures 1 and 2. It was found that immobilization was more effective at higher pH values for all three developed supports. Immobilization is not affected by the solvent used.

Example 6.

As with Example 4, 4'-(6-amino-hexil-amino)-benzamidine was arrayed, both manually and using a printing robot (MicroGrid Total Array System, BioRobotics, England), onto the developed surfaces in an aqueous solution containing a pH 10 phosphate buffer in six different concentrations. The slides were incubated for 2 hours at room temperature in a humidity-controlled chamber in order to prevent evaporation of sample droplets. After sample immobilization the slides were washed, first with water, then using an aqueous solution of 1 x SSC (0.1 M NaCl, 15 mM tri-sodium-citrate), 1% BSA (bovine serum albumin) and 0.2 w/w% SDS (sodium-dodecyl-sulfate), and then with water again. Later, the slides were dried at room temperature and stored at ambient temperature in the dark.

Trypsin was used for a probe molecule, fluorescently labeled with Alexa 647 dye. Amino reactive Alexa 647 carboxylic acid succinimidyl ester (Molecular Probes, USA) was used as a labeling reagent.

Prepared arrays were incubated with 10 μ L of Alexa 647 labeled trypsin (4 pmol) under a glass coverslip for 1 hour at room temperature. Later, the slides were washed with PBS (phosphate buffer, pH 7.0) and then with water. After the slides had dried, they were scanned using a GSI Lumonics, Scannaray Lite confocal laser scanner. Results are shown in Figure 3.

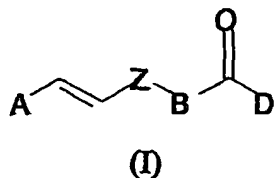
CLAIMS

This invention claims to be:

1./ A method for preparing an active solid structure capable of binding, anchoring or immobilizing biologically active or potentially active organic chemicals by covalent bonds, comprising

a.) a solid support that is prepared in advance, preferably through pre-treatment executed in the presence of strong bases or acids then reacted with a polyamine-functionalized alkoxy-silane derivative, preferably 3-[2-(2-aminoethylamino)ethylamino]propyl-trimethoxy-silane, followed by washing and drying,

b1) the solid support obtained in the procedure described above in Section a.) will be reacted with (I)



— where

Z: = bond or a -CO or -CNR₁ group and group R₁ is an alkyl, aralkyl or aryl group;

B: = bond or a -(CH₂)_n- or -(CH₂)_n-O-(CH₂)_n- or O-(CH₂)_n- or -NH-(CH₂)_n- group;

A: = a branched or unbranched alkyl or substituted aryl or aralkyl group containing 1-5 carbon atoms;

D: = a halogen or -O-A group and

n = 0, 1, 2, ... integer – with a bi-functional reagent, preferably reacted with acryloyl/chloride or 1,4-butanediol diacrylate in the presence of an organic or inorganic scavenger

or

b2) the support prepared using the method described in part (a) is reacted with a bi-functional reagent, preferably 1,4-butanediol diglycidyl-ether or epichlorohydrin, in the presence of an organic or inorganic scavenger containing terminal epoxy groups and an amino-group reactive functional subgroup.

2./ The method described under claim 1, additionally featuring glass, polystyrol or polypropylene chips, preferably glass microscope slides, applied as a solid support.

3./ The active solid support produced using the various methods described under claim 1.

4./ Application of the active solid support described under claim 3 for the production of chemical microarrays and combinatorial chemical microarrays.

5./ A method for the immobilization of what have been termed organic molecules of "low molecular weight", have a size less than 1500 Da, preferably between 250 and 750 Da. This is achieved by anchoring an unmodified drug or drug candidate molecule to the active solid support described under claim 3 by addition of the appropriate functional groups of the drug or drug candidate molecule, preferably executed using a miniaturized robotized binding technique.

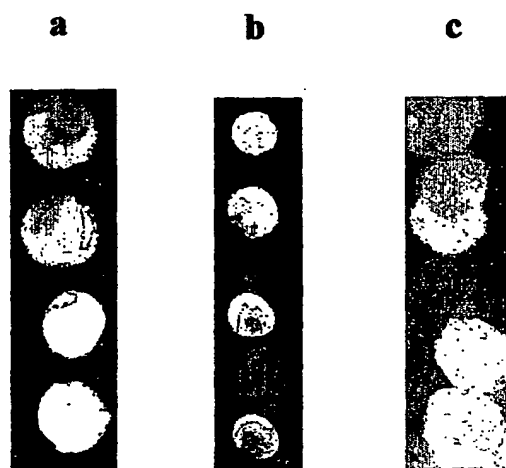
6./ Application of the active solid support described under claim 3 and the microarray produced according to claim 5 for quick execution of agrochemical, biotechnological, biological, or medicinal chemical tests, preferably for studying the interaction between small molecules and proteins.

7./ Application of the active solid support described under claim 3 and the microarray produced according to claim 5 for identification of new proteins, quick analysis of the druggability of proteins, determination of secondary binding sites, identification of the nucleic acids (RNA, DNA) that bind small molecule ligands, identification of protein-protein, protein-DNA, protein-lipid, protein-nucleotide, protein-carbohydrate, protein-vitamine, and protein-metal ion complexes, or identification of ligands and/or metal ions.

8./ Application of the active solid support described under claim 3 and the microarray produced according to claim 5 in biological screening assays, in which microarrays containing immobilized ligands and known or unknown proteins or complexes of these proteins formed by biomolecules are used in either labeled or unlabeled (for example fluorescent) form, or labeled or unlabeled complexes of known or unknown nucleic acids are used, or ions or their complexes are used, or eukaryotic or prokaryotic cells are used, and then the quantity and quality of the bound proteins, nucleic acids, ions, or cells is determined using an appropriate analytical technologies.

9./ Application of the active solid support described under claim 3 and the microarray produced according to claim 5 for determination of anchoring kinetics, balances and kinetic constants, and balance constants.

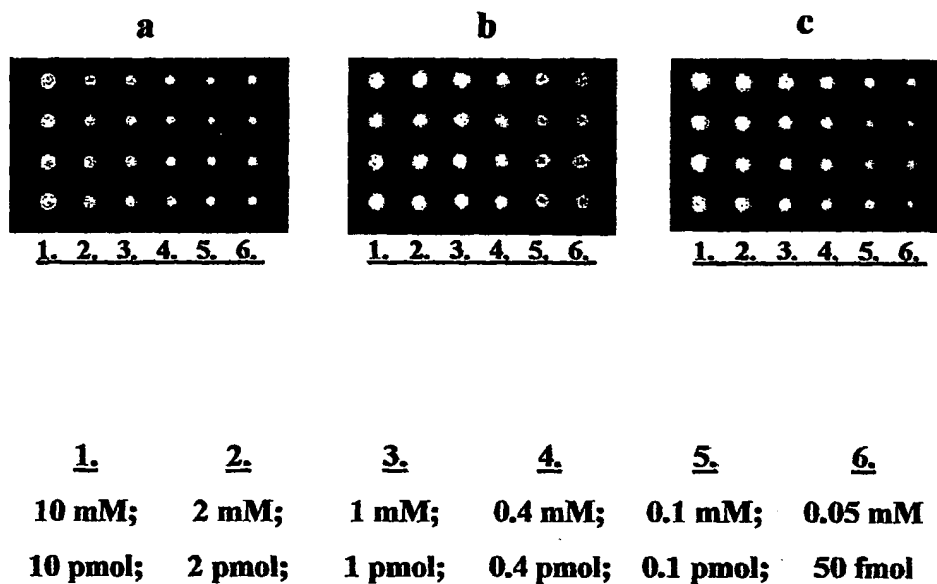
1/3



a.: acrylic surface, b.: hydrophilic epoxy surface, c.: hydrophobic epoxy surface

Fig. 1

2/3



a.: acrylic surface, b.: hydrophilic epoxy surface, c.: hydrophobic epoxy surface

Fig. 2

3/3



1. 2. 3. 4. 5. 6. 1. 2. 3. 4. 5. 6. 1. 2. 3. 4. 5. 6.

<u>1.</u>	<u>2.</u>	<u>3.</u>	<u>4.</u>	<u>5.</u>	<u>6.</u>
20 mM;	4 mM;	2 mM;	0.8 mM;	0.2 mM;	0.02 mM
20 pmol;	4 pmol;	2 pmol;	0.8 pmol;	0.2 pmol;	20 fmol

a.: acrylic surface, b.: hydrophilic epoxy surface, c.: hydrophobic epoxy surface

Fig. 3